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Characterization of calves exhibiting a novel inheritable TNF-α hyperresponsiveness to endotoxin: associations with increased pathophysiological complications

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Elsasser, T. H., J. W. Blum, and S. Kahl. Characterization of calves exhibiting a novel inheritable TNF-α hyperresponsiveness to endotoxin: associations with increased pathophysiological complications. *J Appl Physiol* 98: 2045–2055, 2005. First published January 20, 2005; doi:10.1152/japplphysiol.01050.2004.—A subpopulation of calves, herein termed "hyperresponders" (HPR), was identified and defined by the patterns of plasma TNF-α concentrations that developed following two challenges with endotoxin (LPS, 0.8 μg *Escherichia coli* 055:B5 LPS/kg$^{0.75}$ live body wt) separated by 5 days. The principle characteristic of HPR calves was a failure to develop tolerance to repeated LPS challenge that was evident in the magnitude of the TNF-α concentrations and prolonged severity of pathological sequellae. Whereas calves failing to develop LPS tolerance were identified on the basis of their excessive in vivo plasma TNF-α concentration responses, in vitro TNF-α responses of peripheral blood mononuclear cells isolated from each calf and challenged with LPS or PMA did not correlate or predict the magnitude of in vivo plasma TNF response of the calf. Intentional breeding to obtain calves from bulls and/or cows documented as HPR resulted in offspring displaying the HPR character when similar progeny calves were tested with LPS in vivo, with extensive controls in place to account for sources of variability in the general TNF-α response to LPS that might compromise interpretation of the data. Feed intake, clinical serology and hematology profiles, and acute-phase protein responses of HPR calves following LPS were significantly different from those of calves displaying tolerance. These results suggest that the pattern of plasma TNF-α changes that evolve from a low-level double LPS challenge effectively reveal the presence of a genetic potential for animals to display excessive or prolonged pathological response to LPS-related stress and compromised prognosis for recovery.

cytokine; inflammatory stress; disease susceptibility

INCREASINGLY RECOGNIZED ARE subpopulations of humans and animals that vary in their susceptibility to and recovery from immune stress (3, 73). Cytokine responses to provocative stress challenges modeled by endotoxin (LPS) challenge, as well as active infection, have received great attention as indicators and mediators of both homeostatic and pathophysiological processes in immune response in vivo. While some controversy exists over the importance of measured cytokine levels in disease as prognostic indicators of outcome (41, 61, 64), several lines of evidence suggest that the outcome of some infections may be aligned with plasma levels of cytokines, particularly TNF-α (19, 39, 52, 62, 65–69). Transient bursts of TNF-α production are essential for initiating critical mediator response cascades of other cytokines, arachidonic/eicosapentanoic acid compounds, acute-phase response proteins, nitric oxide, and adrenomedullin in an intricate balance of pro- and counterinflammatory processes (5, 12). However, septic shock (1, 3, 5, 46), chronic tissue wasting (14, 46), and multiple-organ failure (68, 70) have been attributed to an overproduction or chronic production of TNF-α subsequent to the introduction of an immune challenge. A key feature of what is characterized as a normal TNF-α response to a stimulus such as LPS is the development of tolerance to repeated exposures to LPS. Associated with tolerance is a temporally defined reduction in the intensity (e.g., plasma concentration) of the response mediators, as well as clinical signs in successive challenge exposures (38, 69). Several lines of research have equated more severe pathophysiological response in the host when the development of tolerance is compromised (1, 38, 66, 70). The overriding hypotheses of the present research is that *1*) animals that fail to downregulate the intensity of the LPS-TNF-driven proinflammatory response are prone to more severe and/or prolonged pathophysiological response to the challenge, and *2*) the pattern of TNF response to two consecutive LPS challenges can be utilized as a diagnostic tool to predict lineages of animals that may be predisposed to poor recovery prognosis during some proinflammatory diseases.

Data on a genetic predisposition to more overt complications to disease stress as well as ways to effectively predict who or what test subjects would be likely to develop this more intense response are very limited. In this paper, we report the effectiveness of a low-level LPS challenge technique to identify and define characteristics of an inheritable LPS-"hyperresponse" attribute in terms of plasma concentrations of TNF-α-impaired tolerance, exacerbated metabolic and clinical signs, and prolonged recovery time following a double challenge with LPS in calves.

MATERIALS AND METHODS

Animals

All calves used in these studies were produced and reared at the US Department of Agriculture (USDA) Beltsville Agricultural Research Center, Beltsville, MD. For the various experiments, calves were *1*) obtained from either basic Angus × Hereford cows bred with semen purchased from a commercial breeding company, or *2*) derived from

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Angus × Hereford cows sired at the Beltsville research facility by crossbred composite-breed bulls, genetically distinct from Beltsville cows, as developed at the USDA Meat Animal Research Center in Clay Center, NE (MARC-II Composite: ¼ Gelbveih, ¼ Simmental, ¼ Angus, ¼ Hereford), or *3*) derived from planned breedings of bull calves at the Beltsville facility testing positive for hyperresponders (HPR) by the dual LPS challenge protocol on two or more occasions and produced from cows or bulls testing positive as HPRs. All protocols used in these studies were approved by the USDA Beltsville Area Animal Care and Use Committee in regard to appropriate and approved measures of animal comfort, safety, and welfare.

Factors observed by us that affect TNF-α responses to LPS challenge in vivo and contribute to animal-to-animal variability and statistical perturbation were addressed and controlled to the best of our capability. These factors included animal handling and movement (unpublished observations), diet and feed intake (14, 29), subclinical infection (17), reproductive hormonal patterns (43, 55), and diurnal variability (53). Animals were acclimated to procedural handling and used in an unrestrained state free standing in their individual pens. Experimental testing was conducted in either late spring or late fall of a given year with similar ambient photoperiod exposure, and challenges were initiated between 0800 and 0830 on a test day. Calves were fed ad libitum a balanced ruminant diet supplying 14% crude protein and 2.67 Mcal metabolizable energy/kg dried diet (16), with appropriate vitamin and mineral additives as per National Research Council Nutrient Requirements of Beef Cattle (51).

To facilitate ease of bleeding and administration of solutions, a Teflon cannula (Abbocath, Abbott Hospitals, Chicago, IL) was positioned in the right jugular vein of each animal. Cannulas were maintained patent with the instillation of a solution of sterile heparinized (10 U/ml) saline at a volume equivalent to 90% of the void volume of the cannula to minimize inadvertent heparinization of animals, which could compromise the interpretation of the TNF-α response data (10).

All calves used in these studies were tested between 5 and 12 mo of age with management of backgrounding health supported by a standardized program of vaccination, periodic deworming for parasite control, and veterinary oversight. For a given protocol, however, animals were of a uniform age (month of age ± 0.5).

Endotoxin (LPS) Challenge Protocol

The principle dose of LPS chosen was a minimum where all animals challenged were responsive in terms of increased circulating plasma concentrations of TNF-α (at least 1 ng/ml over baseline), the development of slight fever (<3°C over baseline), a brief observable period of increased respiratory and heart rates, and brief hypoglycemia (15). To minimize confounding the interpretation of data because of the body weight (BW) range, the LPS dose was administered as an amount per unit metabolic body size, where the dose is normalized and transposed to BW $(kg)^{0.75}$ (www.anaesthetist.com/physiol/basics/scaling/Kleiber.htm) as proportionate to body surface area (44). We considered the 0.8 $\mu g/kg^{0.75}$ dose [equivalent to the 0.2 μg/kg live weight for a 250 kg calf used in other experiments (11, 13)] as adequate and sensitive for delineation and characterization of TNF-α responses to repeated LPS challenge that would induce tolerance but be devoid of severe pathological consequences or morbidity, which impacted protocol-approved welfare concerns. Where protocol dictated, a higher dose of LPS (9.46 $\mu g/kg\ BW^{0.75}$; ~2 μg/kg live BW) was administered to test the repeatability of the HPR profile during a more intense immune challenge (13). The *Escherichia coli* 055:B5 phenol-extracted preparation (Sigma Chemicals, St. Louis, MO; product number L-2880, lot 58H4076) was considered well suited for the intended purpose where we could capitalize on the endogenous protein content (~1.4%, Sigma Certificate of Analysis) to facilitate the host reaction in terms of LPS protein contributions to the tolerance response as effected through toll-like receptors (24, 37). Similarly, in binding to host lipoproteins, coliform LPS is known to affect specific aspects of LPS-tissue interactions contributing to a component of tolerance separate from that of the major LPS component of the toxin matrix (69) but relevant to our in vivo model.

TNF-α Radioimmunoassay

Concentrations of TNF-α in EDTA-treated plasma and culture media were measured by double-antibody precipitation RIA, as described in Kenison et al. (31). Recombinant bovine TNF-α, obtained from Novartis (formerly Ciba Geigy, St. Aubin, Switzerland), was used to construct the standard curve and iodinated by the Iodogen (Pierce, Rockford, IL) method using Na ^{125}I as the label.

Retrospective Data Evaluation and the Statistical Characterization and Detection of the TNF-α Hyperresponsive Characteristic

Outlier detection and status (e.g., animals with plasma TNF-α concentration changes atypical of the general population) were inferred from an assessment of the normalcy of observation distribution as well as the impact of each individual observation in the set on regression analysis characteristics. The Shapiro-Wilks test (Proc Univariate procedure of SAS; Refs. 56, 58) was applied to describe the statistical distribution where outliers are identified, with particular reference to the presence of kurtosis and skewing from the linearity of a plot of *Z*-score on the *x*-axis vs. response variable on the *y*-axis. To be considered an HPR response, an animal's TNF-α concentration data (peak plasma concentration after LPS or the integrated response area under the time × concentration curve) needed to be greater than 2 SDs away from the population mean in either *challenge 1* or *2*. In addition, outliers, by definition, contribute significantly to a departure from linearity in a graphic representation of response vs. *Z*-score of normally distributed data. For the purposes of establishing and demonstrating a frame of reference and application of this statistical procedure for the present paper, TNF-α challenge response data (area under the time × concentration curve) from a random group of age-appropriate, random-herd calves ($n = 30$) were used to characterize and evaluate a test batch on *E. coli* 055:B5 LPS. Resulting data were subjected to the Proc Univariate statistical procedure. Further characterization of outliers was obtained processing the TNF area response data by regressing the area of response to the first LPS challenge for each animal on the area response from the second LPS challenge and estimating Cook's distance (*D*) and studentized residuals for each observation in the set (Ref. 9; Proc Reg with predicted, residual, and Cook's *D* or "influence" options; Ref. 56). The logic in this approach stemmed from the observed patterned TNF-α responses, wherein a normal response to the second LPS challenge should have been smaller in area magnitude than that to the first challenge and vice versa in HPR. The resulting slopes of trait-associated regressions are essentially opposite in sign, resulting in a major influence skewing the overall regression and normalizing the linearity of the regression when these animals are selectively removed from the data set when processed through Cook's *D* or influence options of the SAS statistical procedure.

Predicting HPR Comparison of In Vitro and In Vivo TNF-α Responses

Part A: Peripheral blood mononuclear cell response to in vitro LPS dose challenge. The objective of this study section was to test whether the magnitude of TNF-α response to specific or nonspecific stimulation of acutely harvested and cultured peripheral blood monocyte cells in vitro had any correlation with the magnitude of a plasma TNF-α response in vivo of the same animal. A preliminary study was performed to ascertain the in vitro LPS dose conditions necessary to obtain a TNF-α response from acutely plated (4 h) and challenged (for 4 h) cells. The acute challenge was considered necessary to minimize artifactual changes in blood cell activities that impact the responsive-

ness of the cells to LPS in terms of TNF-α production (28). For in vitro TNF-α responses to LPS, blood (30 ml in the presence of Alsiever's solution) was collected from 10 random female herd calves. The blood was processed to obtain monocytes, as previously described (6). Briefly, the buffy coat was harvested from the top of the packed red blood cells after centrifugation of samples at 1,850 *g* for 20 min. Cells from the buffy coat were subjected to a ficoll-histopaque density centrifugation (1.077 g/ml) at 800 *g* for 30 min at room temperature. Subsequent centrifugations and washes of banded cells were performed in Alsiever's solution at 180 *g* at 4°C for 20 min. Total cell numbers were determined manually, and cells were plated at 1×10^6 cells per well in 24-well plastic culture plates (Corning Glass Works, Corning, NY) in RPMI-1640 media (Sigma Biologicals, St. Louis, MO) supplemented with 1% fetal calf serum (UBI, Lake Placid, NY). After 4 h, nonadherent cells were washed off the plate with serum-free RPMI. Treatments consisted of 0, 2.5, 5, and 50 μg/ml 055:B5 LPS (Sigma Biologicals) or PMA (100 ng/ml) in 1.0 ml RPMI. Media were harvested after 4 h and stored at −85°C until assayed for TNF-α by RIA. The highest dose (50 μg/ml) was chosen as the test dose based on the magnitude of the TNF-α concentration change in media, the consistency of the response, and its being submaximal in perspective to the still greater response of the cells to the PMA challenge.

Part B: Evaluating and ranking TNF-α secretory response in out-crossbred female calves. Young female calves were produced on site from an outcross of Beltsville Angus × Hereford cows bred to MARC-II bulls with natural service. Twenty-five of the crossbred calves 8–9 mo old were chosen from the cow-calf herd for initial screening purposes. On two occasions, approximately 2 wk apart, 30-ml blood samples were obtained from each calf, and the peripheral blood mononuclear cells (PBMCs) were isolated and plated as described above. In vitro challenges were performed for TNF-α release into media for 4 h on plated cells in triplicate wells from each animal using either 50 μg/ml *E. coli* 055:B5 LPS or PMA (100 ng/ml). Media were harvested and stored at −85°C until assayed for TNF-α. Media from both in vitro LPS challenge times were assayed for TNF-α by RIA and bioassay [WEHI 164 cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-formosan detection of cytotoxicity; Refs. 45, 50]. Media samples from PMA stimulation wells were assayed only by RIA because of reports of PMA downregulating cell membrane receptors for TNF (25), and we judged this to potentially compromise interpretation of bioassay results using the WEHI cells. The WEHI cell cytotoxicity was ascertained as TNF specific when inclusion of the rabbit anti-bovine TNF-α serum (used in the RIA) ameliorated the response, as similarly observed by Nargi and Yang (50), also using this antibody. Media samples were assayed in duplicate in the RIA and in triplicate in bioassays. The absolute concentration values of TNF-α in media from the two in vitro LPS challenge periods, as measured by RIA and LPS bioassay data, were added together and averaged to generate a measure of response termed the "relative response index," and this number was used to rank TNF-α responses across calves from lowest to highest.

For characterization of in vivo TNF-α response, two challenges with LPS (0.8 $\mu g/kg^{0.75}$ iv) separated by 5 days were performed on each of 12 calves. The six calves ranking highest and six ranking lowest in media TNF-α concentration response to LPS (and in parallel to the response obtained with PMA) were selected from the 25 animals tested in vitro to represent the highest and lowest TNF-α response potential, respectively. The 5-day period between LPS challenges was again used to investigate tolerance. Harvested plasma was assayed for TNF-α by RIA. The area under the plasma TNF-α concentration × time curve was calculated by trapezoidal summation and compared with the relative response index ranking generated in the in vitro challenge, as described in *Statistics*.

Extended Characterization of Endotoxin HPR In Vivo

To examine whether the HPR characteristic was stabile and inheritable, a preliminary breeding of HPR female cattle was performed. Three females identified above as HPR, according to their in vivo TNF-α concentration changes following LPS challenges, were bred by artificial insemination with a commercial semen. Each female delivered a calf without complications. The two bull and one female calves from these HPR females were closely monitored from birth. When the calves were 9 mo old, the three animals were conditioned to diet and handling at the barn facility and subjected to the standard double LPS challenge (0.8 $\mu g/kg^{0.75}$ iv). Plasma TNF-α responses to LPS in these calves were compared with those from similarly age- and sex-matched calves from otherwise presumed normal cows bred to the same semen. Because the plasma TNF-α responses to the second LPS challenge in these three calves were greater than the area responses measured following the first challenge (and greater than those of the age- and sex-matched control calves), they were considered HPR. One bull calf (no. 6017) was grown to maturity and returned to the breeding herd for the specific purpose of increasing the number of HPR animals in a specific segment of the herd and thus available for further characterizational testing.

Plasma TNF-α, Feed Intake, Metabolic Profile, Clinical Pathology, and Acute-Phase Response Protein Changes in Confirmed HPR and Normal Calves

The objective of this phase of the study was to characterize the impact of the HPR profile response to repeated higher level LPS challenge on physiological attributes reflective of overall well-being, performance in regard to recovery time, and generalized severity of systemic perturbation. To accomplish this, a 2-yr breeding cycle was developed in which approximately one-third of the breeding female cattle of the Angus × Hereford Beltsville stock were separated from the main herd and exposed to one or more HPR bulls.

For the study, 32 female calves produced from the breeding herd were selected at 4–5 mo of age, including 14 females from the planned HPR breedings. The young calves were brought to the housing testing facility for weaning and standard veterinary care. Calves were dewormed; vaccinated against infectious bovine rhinotracheitis, bovine diarrhea virus, pink eye, and clostridium; and examined for signs of illness. Rectal temperature of all calves was normal. Test animals were acclimated to handling over a 6-wk period and assigned to individual maintenance-feeding pens. Calves were fed ad libitum a balanced diet adequate in protein and energy to result in weight gains averaging ~1.4 kg/day. Water was available continually.

The LPS challenge protocol was initiated in June (referred to in the data as the June study) to avoid confounding effects of excessive summer humidity and temperature, conditions we have observed to unpredictably exacerbate pathophysiological responses to even low-level LPS challenges. For the LPS challenge protocol, each calf was cannulated as described earlier. The 0.8 $\mu g/kg^{0.75}$ LPS challenge was performed on the following day and repeated 5 days later. From the blood plasma obtained at *time 0* and 1, 2, 3, and 4 h post-LPS, TNF-α concentrations were ascertained, the area under the response curves calculated, and the calves ranked from highest to lowest by a simple index (T_{IND}), calculated by dividing the TNF-α area response to the first challenge by the area response to the second challenge. In this manner, equal areas under the time × concentration curve would generate a T_{IND} of 1.0. Similarly, tolerance was suggested where $T_{IND} > 1.0$, and compromised tolerance was represented by $T_{IND} < 1.0$. For the 32 calves, T_{IND} ranged from 3.11 to 0.35. The six calves with the lowest TNF-α T_{IND} were grouped to represent HPR, and the six calves with the highest T_{IND} were chosen to represent the normal or tolerant group for the clinical pathology study. In the selection groupings, T_{IND} of HPR calves averaged 0.62, whereas that of the normal animals averaged 2.39 ($P < 0.005$). Six months later (referred to as the December study), when the cattle were ~10 mo old, these

same 12 subjects were returned to the testing facility and reacclimated to handling, feeding, and movement for weighing purposes. Because these cattle were old enough to have begun to cycle reproductively and therefore express different concentrations of estrogens and progesterone, all cattle used for this experiment were treated with two injections of prostaglandin $F_{2\alpha}$ analog dinoprost tromethamine prostaglandin (Lutalyse, Pfizer-Pharmacia Animal Health, Kalamazoo, MI) to synchronize each to the diestrus stage of the estrus cycle. The first of the two LPS challenges was applied to the cattle 8 days after the second synchronization injection to attain the diestrus phase of the cycle as per package instructions. Control of the reproductive hormone cycle was considered important as a means of reducing the variability in TNF-α response to LPS that relates to the estrogen-progesterone status of animals subjected to LPS challenge (27, 43, 54). For this phase of the experiment, LPS was administered at 9.46 μg/kg $BW^{0.75}$ (equivalent to 2 μg/kg), normalizing the dose administration across the range of BWs (404–514 kg) to test the continuity of the patterns of response at a higher LPS dose.

Feed intake was measured daily for individual animals starting 5 days before the first LPS challenge injection through *day 7* following the second LPS injection. Excess feed was offered fresh daily, and the intake was calculated as the difference between what was offered and what could be weighed back the following morning.

Clinical pathology variables (serum enzymes and hematology) were measured by the Veterinary Diagnostic Laboratory, New York State College of Veterinary Medicine, Cornell University, Ithaca, NY, in samples obtained at the 0-, 7-, and 24-h samples of the two LPS challenge periods. Acute-phase response protein changes were measured in our laboratory using commercially available kits (Phase Range, Tri-Delta Diagnostics, Morris Plains, NJ) for serum amyloid-A (rapid-onset adenosine 5′-phosphosulphate reductase protein) and haptoglobin (slow-onset adenosine 5′-phosphosulphate reductase protein) in plasma validated for use with samples of bovine origin. Plasma TNF-α was measured by RIA, as described previously.

Statistics

In vitro and in vivo response relationships were compared by regression analysis (56). Comparisons between treatment groups for in vivo as well as in vitro data were made using the General Linear Models Procedure (Proc GLM) of the SAS program. In vivo TNF-α responses were assessed and compared between groups by calculating the summated trapezoidal area under the response-profile curve for each animal, as bounded by time and concentration (11), to eliminate the confounding influence of each time point concentration on the preceding and following values, a violation of the independence of observations needed for an analysis of variance based on a GLM of repeated measures. Overall group differences were estimated using the GLM procedure of SAS (56). Aspects of the repeatability of the tolerance (or lack thereof) character of calves over time were further statistically tested by paired-*T* comparisons, where the null hypothesis was accepted, e.g., not different, between the June and December study periods.

RESULTS

Characterization of TNF-α Response Outliers

In Fig. 1*A*, the values represent mean (±SE) plasma concentrations of TNF-α for 30 female calves after two challenges with LPS. These data suggest no clear distinction between the TNF-α responses to the first or second LPS challenge. Figure 1*B* demonstrates the application of outlier detection *Z*-score profile for each animal, where individual statistical outliers are clearly delineated. In this subfigure, the presence of two outliers, indicated by the open circle symbol, impacts the linearity of the plot and skews to the right, where an intended linearity

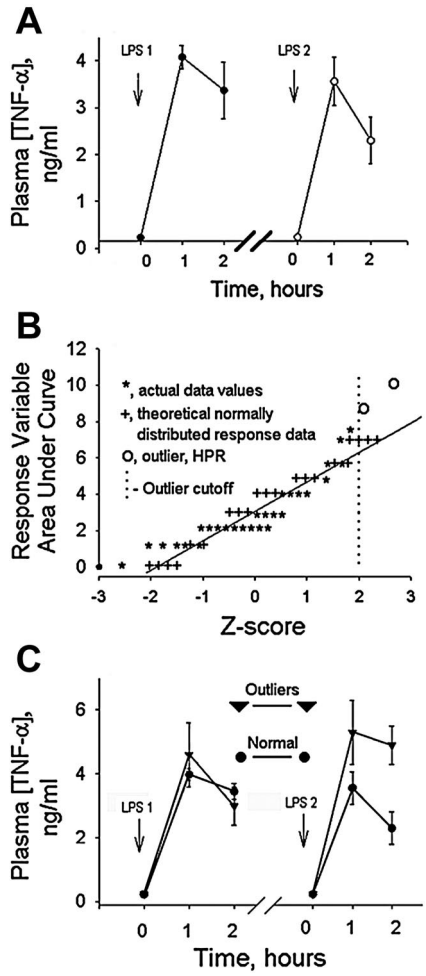

Fig. 1. *A*: plasma concentrations of TNF-α ([TNF-α]) (ng/ml) from calves ($n = 30$) at *time 0* and 1 and 2 h after an intravenous challenge with *Escherichia coli* 055:B5 LPS. LPS challenges were administered 5 days apart. Values represent mean (±SE) plasma [TNF-α]. *B*: assessment of data using the Shapiro-Wilks test of normally distributed data to detect outliers as hyperresponders (HPR) is illustrated by the data (*Z*-score for the response variable in the *Y*-axis) in the 2 open circles, where the deviations from a linear normalized array were significant. *C*: mean plasma [TNF-α] at the times indicated resulting from regrouping data as outliers (HPRs) and normally distributed values ($P < 0.02$).

in the regression of *Z*-score plotted against the *y*-axis data [area under the curve (AUC) in this respect], representing a normally distributed population response, is perturbed. In the regression analysis, the studentized residuals and Cook's *D* for the identified HPR averaged 2.685 and 0.215, respectively, compared with −0.21 and 0.0014 for the same parameters in normal animals, with the same animals confirmed as outliers, as indicated by the *Z*-score analysis. In Fig. 1*C*, regrouped data show that, when outliers are segregated out of the population, HPR TNF-α responses to the second LPS challenge are clearly different than those measured in the main population, with the response characterized as significantly amplified in the two outlier animals vs. those of normal-responding animals.

Comparison of In Vitro and In Vivo Responses and Prediction of In Vivo HPR

The ability of PBMCs to respond to a specific stimulus (LPS, 50 μg/ml) and a nonspecific stimulus (PMA, 100 ng/ml)

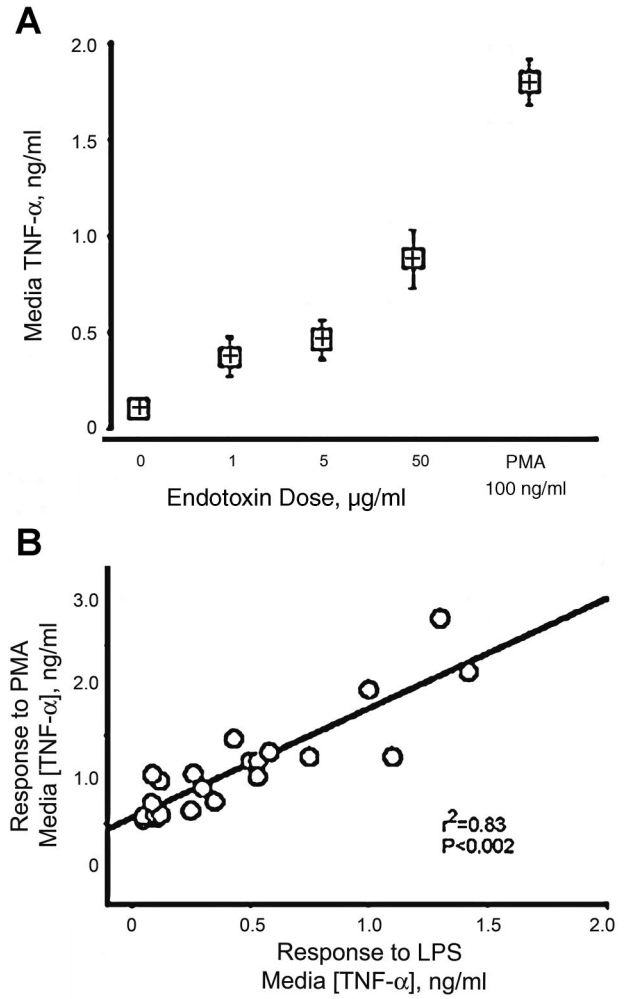

Fig. 2. *A*: culture media [TNF-α] produced by isolated peripheral blood mononuclear cells (PBMCs) challenged with varying concentrations of *E. coli* 055:B5 LPS (μg/ml media) or PMA (100 ng/ml media). Cells were plated at 1×10^6 cells/well and stimulated for 4 h with the respective secretagogue. *B*: the regression relationship between media [TNF-α] from PBMCs treated in vitro with LPS (*E. coli*, 055:B5, 50 μg/ml) or PMA (100 ng/ml) indicated that cells from a given animal responded similarly to each secretagogue, although great variability in response to either secretagogue existed between animals.

is presented in Fig. 2. In a standard construct of a dose-response relationship, data in Fig. 2*A* illustrate that media concentrations of TNF-α generated from the acutely plated and challenged PBMCs increased with increasing concentrations of LPS.

The generation of the relative response ranking was further assessed by challenging acutely plated PBMCs from 25 female cattle with 50 μg/ml LPS or 100 ng/ml PMA. Media concentrations of TNF-α ranged between 0.07 and 1.42 ng/ml with LPS and between 0.11 and 2.32 ng/ml with PMA. Regression analysis demonstrated that the cells' TNF-α responses to each stimulus were similar within an animal (Fig. 2*B*); i.e., cells from calves that presented high media concentrations of TNF-α in response to LPS also had high media concentrations of the cytokine in response to PMA. When the media concentrations of TNF-α as measured by RIA and bioassay from the two separate in vitro challenges were summated and averaged to obtain a relative response index, the absolute numbers ranged between 0.47 and 2.44 ng/ml. When these were placed in rank order from lowest to highest and the populations were selected to represent potential six low and six high in vivo responders, the mean relative response index values averaged 0.59 ± 0.05 and 1.77 ± 0.12 ng/ml for low and high groups, respectively ($P < 0.005$).

Administration of the two LPS challenges to each female calf in vivo resulted in the profile curves depicted in Fig. 3*A*. Average plasma concentrations of TNF-α peaked at 1 h after each LPS challenge and returned to baseline by 4 h. When grouped by the high and low responders predicted from the in vitro relative response index, both groups presented similar profiles for concentrations of plasma TNF-α as a function of time and LPS challenge period. In both selection groups, peak plasma concentration was highly correlated with the area under the concentration × time response curve ($r^2 = 0.967$, $P < 0.005$, data not shown). The mean TNF-α area response of the

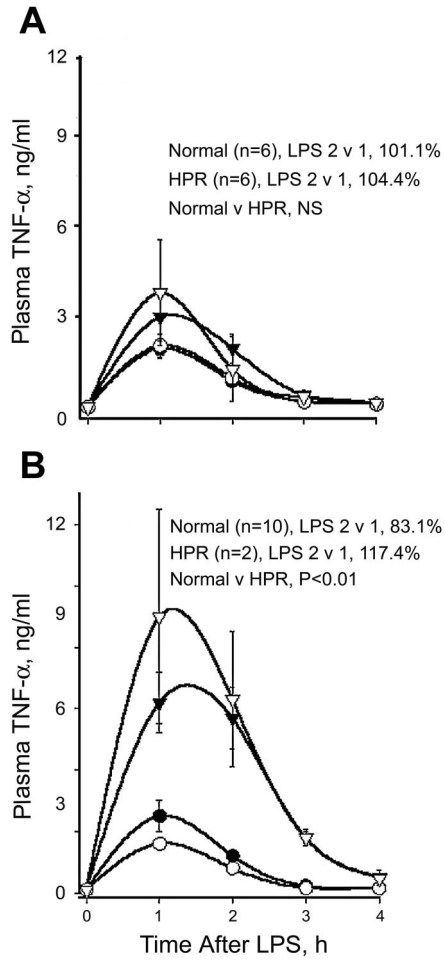

Fig. 3. *A*: mean (±SE) plasma [TNF-α] in calves after 2 in vivo LPS challenges (*E. coli*, 055:B5, 0.8 $\mu g.kg^{0.75}$ iv, bolus) separated by 5 days as grouped by potential for hyperresponse or normal response as predicted in vitro by media [TNF-α] produced by PBMCs of each individual calves in response to 50 μg LPS/ml added to culture media. *B*: retrospective regrouping of the same animals based on measured in vivo TNF-α response patterns to the first and second LPS challenges and determination of true tolerance based on a significant diminution in area TNF-α response following the second challenge. Triangles, potential or actual HPR; circles, potential or actual normal calves; solid symbols, first administration of LPS; open symbols, second administration of LPS. NS, nonsignificant; v, versus.

low-select response group was not statistically different from that of the high-select group, nor was there any indication of a tolerance response, i.e., a smaller area response associated with the second LPS challenge.

Retrospective regrouping of in vivo responses led to the observation that there were indeed animals in both "predicted" response groups that had characteristics of HPR profile. Based on the defining criteria that the HPR TNF-α response to the second LPS challenge should be greater than the response to the first challenge and larger than the response of the average population, the regrouped data of 2 "HPR" and 10 "normal" calves are presented in Fig. 3*B*. The average area responses of the designated normal group ($n = 10$) were 3.32 and 2.78 area units, respectively, for the first and second LPS challenges ($P < 0.05$ for a reduced response in the second challenge). The average responses for the high group ($n = 2$) were 13.5 and 15.8 area units, respectively, for the first and second LPS challenges ($P < 0.05$ for increased response in the second challenge; $P < 0.01$ for low vs. high groups). Based on statistical *Z*-score criteria for identification of outliers, the separation of calves into the respective high- and low-response groups on the basis of magnitude of response to first and second LPS was warranted. Note, when the high responders were factored out, the magnitude of the SEs of the means at each time point was reduced, and the plot of *Z*-score against response regained linearity (data not shown).

Intentional use of an HPR bull to supplement our generalized artificial insemination program for herd replacement animals resulted in an increased number of cattle available to further examine the characteristics of the HPR. Using the in vivo dual low-level LPS challenges (0.8 μg/kg$^{0.75}$) to screen 32 progeny calves at a young age, we designated six as normal and six as HPR, again, based on a calculated index of ranking (T_{IND}) based on the ratio of the TNF-α area response of the first (AUC1) and second (AUC2) challenges. These 12 animals were used subsequently in a test of responsiveness at a higher LPS dose. Data in Fig. 4*A* demonstrate that *1*) the HPR pattern was again present in 19% of this calf population ($T_{IND} < 1.0$). The lack of a demonstratable quality of tolerance was clearly evident in the responses at both LPS doses (Fig. 4*B*). The response for each calf ascertained from the low-dose challenge (June) was maintained over time (repeatable 5 mo later) in the same animals and similarly maintained across the low- and high-LPS doses. The significant correlation of these response data is presented in Fig. 4*C*. When the response data from the June (low-dose LPS challenge) and the December studies (high-dose LPS challenge) are transformed by multiplying the T_{IND} variable for each animal by the difference in the AUC responses for each dual LPS challenge [$T_{IND} \times$ (AUC1 − AUC2)], a rapid visual differentiation of normal and HPR is generated where the transformed responses for HPR are negative values and those of animals displaying tolerance are positive values. The statistical regression of transformed responses from the low-dose challenge on the high-dose responses suggested a significant correlation ($P < 0.02$).

Demonstration of relevant signs of increased pathophysiological response of HPR animals to immune challenge mimicked by the LPS challenges is presented in Figs. 5 and 6 as well as Table 1. With the use of voluntary feed intake and BW change as integrative indexes of overall well-being, the data in Fig. 5*A* suggest that animals designated as HPR consumed less feed after each LPS challenge compared with normal animals but, more importantly, recovered appetite more slowly ($P < 0.03$). When the data were reanalyzed on the basis of normalizing intake changes for each animal as a percentage of mean

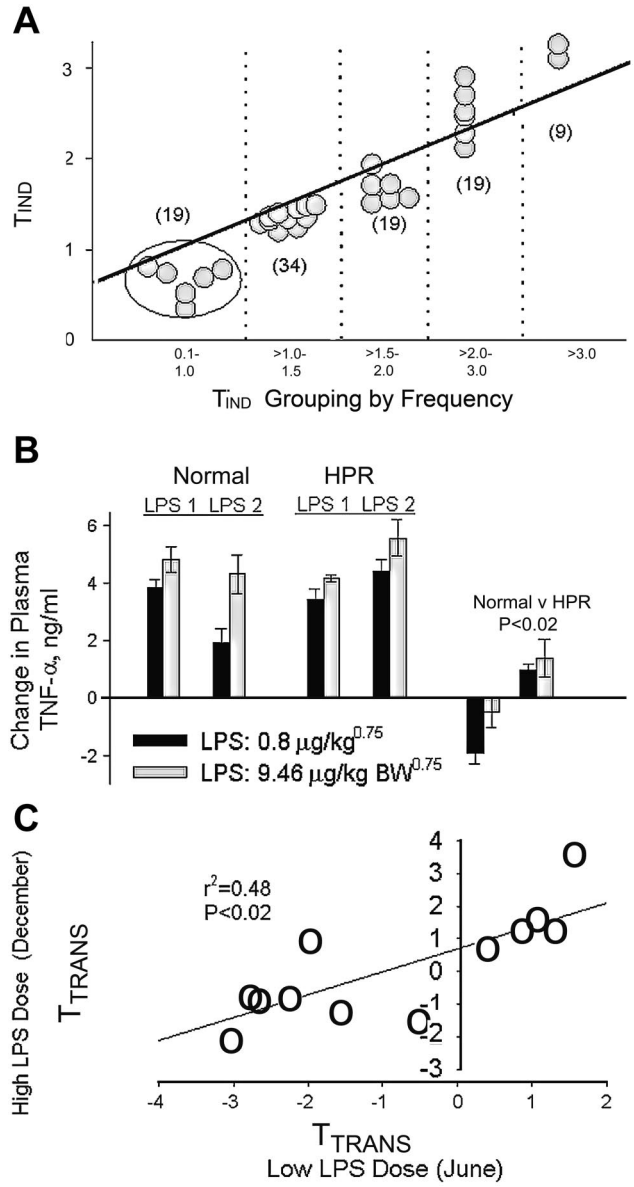

Fig. 4. *A*: HPRs, grouped within the ellipse, were identified as outliers on the basis of their *Z*-score for the response variable, T_{IND}, and associated departure from linearity within the plot of normally distributed data (straight line). Numbers in parentheses are the percentages of the total population. *B*: mean (±SE) incremental increase from *time 0* to the measured peak in plasma [TNF-α] following two LPS challenges (0.8 μg LPS/kg$^{0.75}$) separated by 5 days, conducted with an initial characterization challenge of calves at 5 mo of age and repeated again with calves at 10 mo of age (9.46 μg LPS/kg$^{0.75}$). Data are grouped for HPR and normal TNF-α response in vivo ($n = 6$/group) based on the characterization of response to the 0.8 μg LPS/kg$^{0.75}$ dose from June testing. In the graph subsection labeled "Normal v HPR", values represent mean differences in peak plasma [TNF-α] between *challenge 1* and *challenge 2* at each LPS dose. *C*: regression analysis of in vivo TNF-α responses [$T_{IND} \times$ (AUC1 − AUC2), where AUC1 and AUC2 are first and second areas under the curve, respectively] indicated that the characterization of calves as HPRs or normal responders to the two LPS challenges was consistent over time within animal and across doses. BW, body weight; T_{TRANS}, transformed responses.

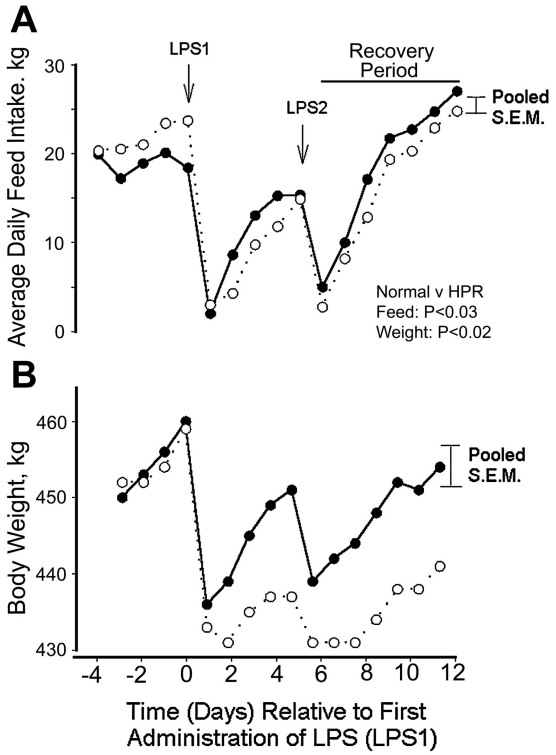

Fig. 5. *A*: average daily feed intake weights in normal ($n = 6$) and HPR ($n = 6$) calves challenged twice, 5 days apart, with 9.46 μg/kg$^{0.75}$ *E. coli* 055:B5 LPS at times indicated. *B*: average daily live BW change of the calves described in *A*. Error bars indicate the pooled variance derived from the general linear statistical model applied to the data. ○, HPRs; ●, normal calves.

ad libitum intake for the 5 days preceding the first LPS challenge, the HPR cattle consumed 31% less feed than the normal animals ($P < 0.02$). Similarly, the BW loss (Fig. 5*B*) that occurred after LPS challenge (a composite effect of diminished feed intake, increased loss of gut contents, and fluid volume loss and dehydration) was more severe in HPR animals and significantly slower in recovery ($P < 0.02$).

Clinical variables as summarized in Table 1 indicate significant changes over time in all variables measured as a result of LPS administration per se with regard to which of the measured variables were impacted. For the most part, few traditional serological chemistry markers were different between HPR and normal calves. A substantially greater impact of the LPS challenge on liver and muscle integrity was suggested in serum enzyme profiles of sorbitol dehydrogenase, aspartate transcarbamylase, and creatine kinase. The most consistent difference between HPR and normal calves was evident in the hematology profile changes and distribution patterns of white blood cells, segmented neutrophils, and lymphocytes. Especially important in this regard was the continued significant leucopenia present in the HPR calves following the second LPS challenge.

Data in Fig. 6, however, reveal that both slow-onset (haptoglobin) and rapid-onset (serum amyloid A) acute-phase response protein changes were significantly more perturbed in HPR animals compared with calves that developed tolerance to repeated LPS challenge. Especially noticeable was the continued elevation of both acute-phase proteins in HPR (*time 0* at the application of the second LPS challenge), as well as a maintained augmented response in acute-phase protein production throughout the secondary challenge ($P < 0.01$).

DISCUSSION

The ramifications of the present data suggest that HPR animals constitute a population at high risk for exacerbated pathophysiological responses to immune challenges. The impact of this could affect *1*) a need for increased veterinary/medicinal intervention, *2*) increased production and management costs for these animals to recover, *3*) a potential for increased antibiotic use if this characteristic impacts susceptibility to bacterial and viral infection complexes typical of production cattle, and *4*) a higher environmental impact associated with inefficient nitrogen utilization and digestive processes and potential for increased zoonotic transfer human health risk.

In three previously published experiments conducted to characterize the effects of hormonal status, feeding level, dietary protein content, or arginine infusion (11, 14, 30) on the TNF-α response to LPS, the data indicated that the TNF-α responses peculiar to a very limited number of animals could have a major impact on the statistical level of significance that was observed for a given treatment effect and, therefore, the interpretation and conclusions of the study. The impact issue was a matter of animal-to-animal variability, with attention brought to specific animals that could be clearly labeled as statistical outliers and now recognized as the unique subpopulation of animals termed herein HPR. Data in the present paper define and substantiate the existence of calves with a hyperresponse attribute from different sire-dam pairings and confirm the second attribute of hyperresponsiveness, that being the compromised development of LPS tolerance measurable 5 days after the initial endotoxin challenge. As observed previously, these animals are few in number. However, when we have defined and eliminated as many sources of variation as we could account for that affect the TNF response to LPS and perform the LPS challenge protocol, we find that the HPR

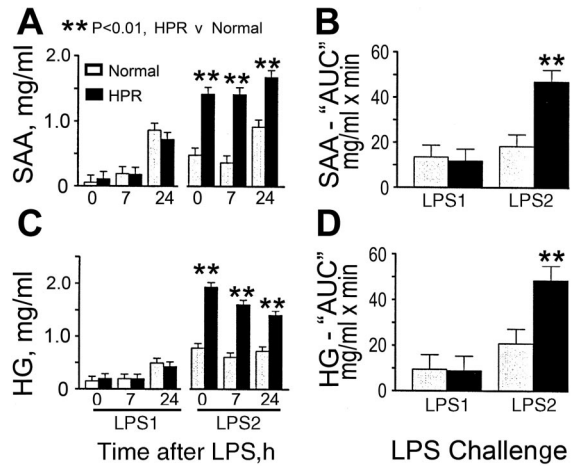

Fig. 6. Changes in plasma acute phase response proteins in HPR and normal animals before and after 2 challenges of LPS (*E. coli* 055:B5, 9.46 μg/kg$^{0.75}$) separated by 5 days. *A* and *B*: plasma concentrations (means ± SE) and area under the time × concentration curve responses for serum amyloid A (SAA) from normal (open bars) and HPR (solid bars) calves ($n = 6$/group), respectively. *C* and *D*: similar acquired and representative grouping data, respectively, for haptoglobin (HG). **$P < 0.01$, HPR vs. normal.

Table 1. *Effects of repeated endotoxin challenge (Escherichia coli 055:B5; 9.46 g/kg body wt$^{0.75}$ and TNF-α response type on common clinical chemistry variables and blood cell counts at 0, 7, and 24 h relative to each of two administrations of the LPS 5 days apart*

Parameter	Type	LPS *Challenge 1*			LPS *Challenge 2*			Pooled SE
		Time 0	+7 h	+24 h	*Time 0*	+7 h	+24 h	
White blood cells, $10^3/\mu l$	Normal	8.8†	2.4†	10.5	9.0	4.2†	14.1	0.7
	HPR	11.1	4.2	8.7	9.3	1.9	11.0	
Segmented neutrophils, $10^3/\mu l$	Normal	3.4	0.8*	6.8	3.5	1.9*	8.8	0.8
	HPR	2.0	0.2	5.1	2.6	0.3	5.3	
Lymphocytes, $10^3/\mu l$	Normal	6.5‡	1.9†	2.8	5.1	1.9	5.7	0.4
	HPR	8.9	3.8	3.4	6.3	1.5	5.1	
Sorbitol dehydrogenase, U/l	Normal	21.5	57.1	80.0	17.7	81.8	67.6	14.0
	HPR	22.6	72.0	118.0*	15.6	118.0*	84.6	
Aspartate transcarbamylase, U/l	Normal	68.8	301.9	249.0	81.6	322.3*	183.4	34.7
	HPR	75.8	413.0	410.6	82.0	388.8	275.6	
Creatine kinase, U/l	Normal	189.2	340.3	481	143	209	270	28.4
	HPR	194.7	384.2	622*	233*	272	372*	

Values represent least squares means of 6 calves per type generated from the General Linear Models procedure. Normal, normal calves with TNF-α tolerance to a second LPS administration; HPR, hyperresponders, calves displaying a repeatable pattern of blunted or no TNF-α tolerance to the second LPS challenge. Measured hematocrit, alkaline phosphatase, γ-glutamyl transaminase, creatinine, plasma urea, and albumin concentrations were not different between HPR and normal cattle at the time points tested. Other than albumin, each variable increased significantly following the LPS challenges with peak concentration or unit activity peaking in the 7-h post-LPS samples. *$P < 0.05$; †$P < 0.02$; ‡$P < 0.005$: means for a stated time within an LPS challenge period are significantly different between tolerance types. See text for details of parameter responses integrated over time.

profile for a given animal is repeatable over time, can be genetically passed across generations, and is consistent when low and moderate doses of LPS are used for the challenge.

Single observations of perturbations in biological response to a challenge are often ignored as inconsequential noise in parametric statistical analysis of data. However, multiple observations of the TNF-HPR-physiological response to repeated LPS raised questions in our minds regarding whether this overresponsiveness in TNF-α and associated biological response pattern were repeatable within animals and across populations. When we intensified deliberate attempts to produce calves with this HPR characteristic by *1*) breeding hyperresponsive cows back to random commercial bull semen, or *2*) introducing HPR-positive bulls to the herd, we increased the proportion of calves that presented plasma TNF-α and clinical responses consistent with the definition of the HPR. For example, we bred HPR cow no. 3018. That year's calf, a bull calf (no. 6017), was weaned early at 10 wk of age as a result of needed exceptional veterinary care associated with chronic diarrhea, a rectal prolapse, and recurrent coccidiosis, which eventually resolved with repeated Ivermectin treatment. This bull calf (no. 6017) was tested starting at 4 mo of age for the presence of the aberrant pattern of TNF-α response to the standard double 0.8 $\mu g/kg^{0.75}$ LPS challenge. Repeated challenges on this bull at 9 and 14 mo showed similar HPR characteristics. Further breedings of HPR cow no. 3018 and HPR cow no. 3022 were problematic, and several times these cows failed to conceive by either artificial insemination or natural service. Consequently, they were removed from the active breeding herd. However, a second calf (no. 8063) of cow no. 3018, produced 2 yr later, again tested positive as an HPR with the attributes of high first response to LPS and a lack of tolerance to a second LPS challenge. In addition, when HPR bull no. 6017 was introduced into a subpopulation of the general breeding herd cows, >70% of the calves sired by this bull tested positive, by statistical outlier analysis, for the HPR characteristic, with the remainder displaying plasma TNF responses visibly abnormal, for example with extraordinarily high (2- to 3-fold higher than normal calves) TNF-α responses to both challenges. Calves born to the same cows bred to commercial semen 1 yr earlier were not HPR positive.

The "gold standard" for identification of HPR animals would appear to be the in vivo double LPS challenge. Although invasive, a standardized protocol is presented that minimizes the impact of LPS on the animal and provides a definitive result. In this regard, the utility for this testing procedure might be best directed toward breeding stock, for example in the cattle industry, where the presence of the trait in a given animal might indicate a management decision to not further breed with this animal. An in vitro determination of the HPR would facilitate the screening of numerous animals to identify genetic HPR carriers simply by being relatively noninvasive. However, identification by in vitro screening of calves that overreact to LPS is more complicated and problematic than attempts here could resolve. Initial interest in screening for the phenomenon was derived from observations that peripheral mononuclear cells are a principle source of TNF-α (6, 28, 62), and the excessive response of these select calves might have been related to increased per-cell release of cytokines.

In vitro stimulation tests with LPS and PMA in our studies were similar in magnitude. The fact that PBMC responses to a specific (LPS) and general (PMA) secretagogue were similar within animals and between two separate in vitro challenge experiments suggests that the present in vitro results also are repeatable and not simply an artifact of a one-time event. However, the utility of this approach to define the response characteristics of interest here was limited.

In vitro and in vivo responses were poorly correlated and without statistical significance. The lack of correlation between TNF-α measurements and rankings in response to in vitro and in vivo stimulations suggests that elements in the regulation of the hyperresponse are substantially more complicated than can be assessed by the simple amount of cytokine released by individual cells in culture. Certainly, in vitro, it is impossible to

mimic the extent of additional factors and homeostatic responses present in vivo that impact and shape the magnitude and duration of cytokine responses to LPS. The PBMC response to LPS in vitro becomes more difficult to interpret because of a dynamic change in cellular sensitivity to LPS as monocytes begin to evolve to macrophages following the sensitizing first exposure to LPS (29), a factor limiting the utility and interpretation of a repeated LPS challenge in vitro for the present intended purpose. Additional response elements needing consideration and clarification include the production rate and tissue source of TNF-α, clearance dynamics, presence of circulating soluble receptor and LPS binding protein(s), plasma membrane receptor distribution and activity, and the whole host of interactions that contribute to the establishment of the tolerance phenomenon. Thus the propensity to model this level of cell-to-cell interaction is largely beyond the scope of available methodology, even in cell coculture technologies, and is fraught with difficulties in extrapolating to the in vitro situation.

The overproduction and chronic production of TNF-α in experimental and actual disease states have been recognized early on as an effector of many adverse metabolic effects of sepsis stress (6, 19). When direct and provocative bolus LPS challenges are imposed that result in measurable increases in concentrations of TNF-α in peripheral plasma of cattle, the TNF-α response is brief in duration, peaking between 1 and 2 h after challenge and often returning to baseline by 4–6 h (11, 32, 33). Even when LPS is administered as a prolonged infusion in the cow (18) or in the sheep (59), the TNF-α response is usually terminated following the peak at 2–3 h, despite the continuation of the LPS infusion. Termination of the production of TNF-α may be a stabilizing homeostatic process associated with survival. This may be mediated, in part, by the increases in ACTH (7, 57), glucocorticoid (11), and interleukin (36, 62) secretion, concomitant with responses to LPS, prompting feedback downregulation of TNF transcription and translation (5, 42). Furthermore, the glucocorticoid response may be protective in that cytotoxic effects of TNF-α are decreased with glucocorticoids (63). When tested by the repeated LPS challenge in vivo, most noticeable for each HPR animal was the more rapid onset and severity of pulmonary signs, marked hypoglycemia (<20 mg/dl plasma glucose), and hypocalcemia. When recumbent, these calves responded well to intravenous water containing sodium, potassium, magnesium, chloride, gluconate, and lactate plus intramuscular dexamethasone and banamine.

Severe consequences of high-circulating peak levels of TNF-α may not be as much a direct factor in the pathogenesis of endotoxemia as the duration of exposure to factors downstream from the TNF-α event, such as thromboxane-prostacyclin imbalances (59), elements of the nitric oxide-superoxide cascade (8, 13, 35), and the host of interactive factors that culminate in irreversible multiple-organ failure (64, 73), or the cell function disruption associated with the complement-mediated membrane attack pathway (12). Certainly, higher plasma levels of TNF-α foster longer duration of proinflammatory action simply based on the time needed to clear more TNF-α from the tissues and circulation. However, the data from measurement of the acute-phase response proteins serum amyloid-A and haptoglobin corroborate this assessment of prolonged integrative response beyond that of TNF-α alone in that these proteins are reflective of events progressing over hours and days. Likewise, from the simple standpoint that voluntary appetite and weight loss and replenishment are reflective of an animal's overall systemic response to a health challenge, the data illustrate that calves displaying poor development of proinflammatory tolerance suffer a prolonged recovery time during which they may become more prone to heightened pathophysiological response to additive, opportunistic sources of immune stress (12, 17).

Tolerance to repeated administrations of LPS has been observed and characterized since 1947 (2). More recent information on LPS tolerance suggests that *1*) there are multiple types of tolerance, which have characteristics dependent on the time after LPS challenge; *2*) there are multiple regulatory sites that impact on tolerance, with particular emphasis on gene promoter sites with nuclear factor-κB regulatory sites; *3*) tolerance can be apparent in several different physiological forms independently in terms of fever response, cytokine response, and hemodynamic response; *4*) different forms of tolerance and therefore different levels of regulation may exist as a function of time after LPS exposure; *5*) cross-tolerance to LPS may be elicited with direct administration of cytokines; and *6*) a portion of the downregulation in TNF response is associated with aspects of glucocorticoid feedback on cytokine gene transcription (4, 5, 20–23, 30 34, 70, 72), and most recently the observation that tolerance is strongly dependent on the activity of SH-2-containing inositol phosphatase in modulating nuclear factor-κB (68). Other researchers have considered negative feedback effects of interleukins-1, -6, and -8 (25, 67) to be important modulators of tolerance in the associations between repeated LPS exposures and the downregulation of TNF-α and the resultant cascade of oxidative stress effectors. Similar in vivo studies in large domestic species are difficult to perform because of the availability and affordability of specific recombinant cytokines, lack of appropriate ELISA or RIAs for specific cytokines, and inherent technical difficulties in measurement of cytokines in ruminant plasma by bioassay (50).

In this context, the tolerance response also has been described as a survival mechanism to limit inappropriate adverse pathophysiological consequences of the whole body cytokine response to endotoxemia and septic shock (1, 32, 66, 70). Thus the inability of the cattle to downregulate cytokine response to repeated exposure described herein appears to be a risk factor that correlates with a quantifiably heightened severity of particular clinical signs and acute-phase response proteins elaborated with a subsequent exposure to LPS or an immune challenge (1, 52, 61, 64, 73).

There are few data that address the tolerance response to LPS in large domestic animals. The presentation of LPS tolerance in ruminants is particularly intriguing in that the fluids in the functional rumen constitute a sea of mixed endotoxins (47–49). In fact, the quality and quantity of rumen LPS vary in association with feed composition (47, 49). Thus a potential complication to restoring homeostatic balance during bacteriemia and sepsis in HPR-type cattle might be associated with unique tolerance characteristics particular to the constant enterohepatic exposure to these endogenous endotoxins and diminished capacity to compensate for situations that challenge the system further. For example, evidence exists to suggest that endotoxins are taken into the bloodstream in association with

compromised bowl integrity commonly encountered during viral inflammation as well as trauma (26, 40, 48).

As the majority of studies investigating tolerance are associated with rabbits, rodents, and in vitro-type challenges, there are few data that can accurately describe the temporal relationship between timing of LPS exposure and the manifestations of early- and late-phase LPS tolerance in cattle. From an experimental standpoint, the presence of HPR and the presence and duration of LPS tolerance should be considerations in the appropriateness of statistical designs utilizing the switchback, reversal, and Latin-square models, and studies of dose-response protocols calling for LPS and drug testing. The tolerance factor may lead to statistical confounding of treatment and time, and inability to resolve main effect variables, aspects particularly important in drug testing. The physiological ramifications of the hyperresponsive attribute in terms of the type of infection (viral, bacterial, parasitic) obviously remain to be elucidated.

The ability to predict the intensity of response to a disease challenge in food production animals in particular has merit in that *1*) breeding programs could use the response data to identify a genetic propensity for a problem to develop in specific lines of animals; *2*) anticipated natural stresses that occur at birth, parturition, weaning, transport, shipping, or intensive competition in the case of sport animals could be anticipated and dealt with prophylactically; *3*) therapy, on an individual basis, might be better matched with capacity for response; and *4*) the chance for indiscriminate use of antibiotics, development of resistance with such practices, and the introduction of drug residues into the food chain could be lessened.

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DISCLOSURES

Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee, warranty, or an endorsement by USDA and does not imply its approval or usage to the exclusion of other products that may be suitable.

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